Definition of a Sequence, RYVVLPR, within Laminin Peptide F-9 that Mediates Metastatic Fibrosarcoma Cell Adhesion and Spreading

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ABSTRACT

A synthetic peptide from the inner globule of the B1 chain of laminin, termed peptide F-9 (RYVVLPRYCEFKGMNYTVR; residues 641-660), has been shown to have heparin-binding and cell adhesion-promoting activities for diverse cell types (Charonis et al., J. Cell. Biol., 107: 1253-1260, 1988). In this study, the metastatic murine fibrosarcoma cell line, UV-2237-MM, adhered and spread on surfaces coated with laminin and peptide F-9 in a concentration- and time-dependent fashion. Cells migrated toward laminin in Boyden microchemotaxis chambers but not toward peptide F-9. However, exogenous soluble peptide F-9 inhibited both the adhesion and migration of cells toward laminin. Polyclonal antibodies raised against peptide F-9 were capable of inhibiting laminin-mediated cell adhesion and migration. Peptide F-9 is located 265 residues from CDPGYIGSR, another sequence on the B1 chain of laminin which has been reported by others to promote cell adhesion (Graft et al., Cell, 48: 999-1006, 1987). In contrast to peptide F-9, various control peptides including CDPGYIGSR did not promote the adhesion, spreading, or migration of the UV-2237-MM fibrosarcoma cells. In addition, neither exogenous peptide CDPGYIGSR nor antibodies raised against peptide CDPGYIGSR were capable of inhibiting laminin-mediated cell adhesion or migration. These results indicate that peptide F-9, but not peptide CDPGYIGSR, represents a major fibrosarcoma cell adhesion-promoting domain on intact laminin. A series of overlapping peptides were synthesized which contained various portions of the parent peptide F-9. The use of these peptides in cell adhesion assays demonstrated that the sequence RYVVLPR from the amino terminus of peptide F-9 was essential for cell adhesion-promoting activity.

INTRODUCTION

Laminin is a large (850 kDa) basement membrane glycoprotein which regulates the adhesion, spreading, migration, growth, and phenotypic expression of various normal and transformed cell types (1–3). By rotary shadowing and electron microscopy, laminin derived from the murine EHS tumor is cruciform shaped with two globular domains near the ends of the three short arms and one large globular domain at the distal end of the long arm (4, 5). Digestion of laminin with proteolytic enzymes followed by purification of fragments has led to the further localization of some of the biologically active domains of laminin, including several distinct cell adhesion and heparin-binding domains within the protein. For example, certain tumor cells bind to a laminin fragment composed of the intersection of the cross (6, 7), rat hepatocytes adhere and spread on the short arm of laminin opposite the long arm (7), and neurite outgrowth and murine myoblast adhesion is promoted on laminin fragments derived from the distal part of the long arm (8, 9). Monoclonal antibodies have also proven useful to more precisely map certain domains of laminin which promote neurite outgrowth (10), tumor cell adhesion (11), and heparin binding (12).

In recent studies, several laminin receptors have been described for various cell lines, including a 67-kDa receptor isolated from normal and malignant cell lines (2, 6, 13–21), a 110- and 180-kDa protein isolated from neuroblastoma-glioma cells (22), a 120-kDa glycoprotein isolated from various cell lines (23), 56 and 66-kDa receptors isolated from skeletal muscle (24, 25), and 120- and 150-kDa integrins from human glioblastoma cells (26), human osteosarcoma cell line MG-63 (27), several chicken cell types (28), platelets (29), and neuronal cell line PC12 (30, 31). These findings suggest that different cell types adhere to specific domains of laminin based on their various cell surface receptors.

Recently, the amino acid sequences of the B1, B2, and A chains of EHS and human laminin have been determined from the nucleotide sequence of complementary DNA clones (32–38). A synthetic peptide, F-9, which was derived from the B1 chain of laminin near the inner globule of the short arm (residues 641–660) was found to have biological activity (39). This peptide (RYVVLPRYCEFKGMNYTVR) binds heparin and promotes the adhesion of various cell lines including melanoma, fibrosarcoma, glioma, and pheochromocytoma, as well as bovine aortic endothelial cells.

In the current study, the adhesion-promoting activity of peptide F-9 toward a highly metastatic murine fibrosarcoma cell line UV-2237-MM was examined. This cell line was selected due to the ability of laminin to specifically promote its adhesion (40) and the ability of fragments of laminin from the cross-region that contain the peptide F-9 sequence to inhibit experimental lung metastasis when these cells were preincubated with the fragments (40). The results indicate that peptide F-9 represents a major cell adhesion-promoting determinant on intact laminin, since peptide F-9 specifically promoted cell adhesion and spreading. Exogenous soluble peptide F-9 and polyclonal antibodies against peptide F-9 inhibited laminin-mediated cell adhesion as well as laminin-mediated cell migration. However, peptide F-9 did not directly promote the migration of the cell perhaps due to the nature of the migration assay utilized.

Another synthetic peptide, CDPGYIGSR, derived from the B1 chain of laminin near the intersection of the cross (residues 925-933) has been shown to promote the adhesion of human fibrosarcoma HT-1080 cells and Chinese hamster ovary cells (41) and a variety of epithelial cells (42). However, the cells attached to CDPGYIGSR have not been observed to spread on surfaces coated with this peptide (42). The peptide...
CDPGYIGSR has also been reported to bind to a 67-kDa laminin receptor (42), to inhibit experimental metastasis following in vitro pretreatment of cells (43), and promote the migration of B16F10 melanoma cells (41, 44). In this study, peptide CDPGYIGSR was used as one of the control peptides in parallel experiments with peptide F-9 and was found to be inactive in promoting the adhesion, spreading, or migration of UV-2237-MM fibrosarcoma cells.

Further studies were performed to define the functionally active region of peptide F-9, i.e. the minimal amino acids required for peptide F-9 to retain its cell adhesion-promoting activity. Direct cell adhesion assays were conducted using peptides which made up only certain regions of peptide F-9. The heptapeptide, RYVVLPVR, with the amino terminus of peptide F-9 was found to be active at directly promoting cell adhesion. In addition, when smaller synthetic peptides were used to inhibit laminin- or peptide F-9-mediated cell adhesion, the sequence which still retained inhibitory activity was comprised of the amino acids RYVV. These results indicate that the peptide F-9 domain of laminin, and in particular the sequence RYVV, plays an important role in promoting the adhesion of UV-2237-MM fibrosarcoma cells to laminin.

MATERIALS AND METHODS

**Proteins and Synthetic Peptides.** Laminin was isolated from the EHS tumor as described previously (45) with minor modifications. Briefly, tumor was homogenized in 3.4 M NaCl-0.01 M phosphate buffer, pH 7.4, with 50 μg/ml of the protease inhibitors phenylmethysulfonyl fluoride (Sigma Chemical Co., St. Louis, MO) and p-hydroxymercuribenzoate (Sigma Chemical Co.), washed twice with the same buffer, and then extracted overnight with 0.5 M NaCl-0.01 M phosphate, pH 7.4, and 50 μg/ml of the protease inhibitors. The salt concentration was raised to 1.7 M followed by stirring overnight at 4°C and then spun at 15,000 rpm for 15 min. Laminin was precipitated from the supernatant overnight at 4°C with 30% saturation ammonium sulfate. The precipitate was resuspended in 0.5 M NaCl-0.01 M phosphate, pH 7.4, and dialyzed against the same buffer. Aggregates were removed by ultrafiltration at 40,000 × g for 1 h at 4°C. Laminin was isolated from the supernatant by gel filtration chromatography on Sephacryl S-300 (Pharmacia Fine Chemicals, Piscataway, NJ) (2.6 × 100-cm column) in the 0.5 M NaCl buffer, where it eluted just after the void volume. The laminin solution was concentrated by evaporation while in a dialysis bag, dialyzed against PBS, and stored at −70°C. Type IV collagen was isolated from the EHS tumor (46) and fibronectin was purchased from Sigma Chemical Co.

**Isolation of Peptides.** F-9 was found to be active at directly promoting cell adhesion. In addition, when smaller synthetic peptides were used to inhibit laminin- or peptide F-9-mediated cell adhesion, the sequence which still retained inhibitory activity was comprised of the amino acids RYVV. These results indicate that the peptide F-9 domain of laminin, and in particular the sequence RYVV, plays an important role in promoting the adhesion of UV-2237-MM fibrosarcoma cells to laminin.
not reduce aldehydes or ketones at neutral pH but it does reduce Schiff’s bases. A 5 to 1 m ratio of [3H]formaldehyde to amide groups in the synthetic peptide was used. Sep-Pak C-18 cartridges (Millipore) were used to isolate and purify the radiolabeled peptide. Briefly, a Sep-Pak C-18 cartridge was preswollen by rinsing it with acetonitrile followed by water and then equilibrated with 100 mM Hepes, pH 7.4. The radiolabeled peptide was then loaded onto the Sep-Pak C-18 cartridge with a disposable syringe. The Reacti-vial was rinsed twice with 200 µl of buffer and the rinses were added to the C-18 column. The cartridge was washed with 0.1 M sodium phosphate buffer, pH 7.2, at a flow rate of about 1 ml/min, until all unbound label was washed through the column. The peptide was then eluted with 50% acetonitrile/0.1% trifluoroacetic acid/49.9% water. Aliquots were measured for radioactivity in a scintillation counter. The radiolabeled peptide was then diluted with 30 µl of water and lyophilized. Prior to use, the radiolabeled peptides were dissolved in distilled water.

In order to quantitate the amount of each peptide adsorbed to the wells after drying down and rinsing, the radiolabeled peptides were diluted in PBS to concentrations ranging from 2 to 200 µg/ml. Fifty µl of these solutions were added to each well, in triplicate, giving a total of 0.1-10 µg of peptide/well. The plates were incubated overnight at 29°C to adsorb the peptides in the wells. Wash buffer (200 µl of 2 mg/ml BSA in DMEM containing 20% FCS) was added and incubated for 2 h at 37°C. The wells were then rinsed three times with 200 µl of this wash buffer at room temperature. Lysis buffer (200 µl of 0.5 M NaOH, 1% sodium dodecyl sulfate) was then added to each well and incubated for 30 min at 60°C. The radioactivity was removed and counted in a scintillation counter.

Cell Adhesion Assays. The direct adhesion of cells to protein- or peptide-coated surfaces was performed as described previously (11, 39). Briefly, radiolabeled cells were added to 96-well microtiter plates coated with various concentrations of synthetic peptides, laminin, or BSA for various lengths of time as shown in the figure legends. After the incubation period, loosely or nonadherent cells were removed by washing the wells three times. Adherent cells were solubilized and quantitated in a scintillation counter.

Spreading of the adherent cells was evaluated by performing adhesion assays similar to those described above. Twenty-four-well tissue culture plates (Becton Dickinson and Co., Lincoln Park, NJ) were coated with 300 µl of laminin, synthetic peptides, or BSA at 100 µg/ml and then blocked with 2 mg/ml BSA in PBS. A cell suspension (300 µl of 5 × 10^4 cells/ml) was added to each well, and after a 2.5-h incubation, nonadherent cells were aspirated out of the wells. Adherent cells were fixed with 2% glutaraldehyde in PBS at 23°C for 1 h. The glutaraldehyde was then removed and the cells were stained by two different techniques. For photographic purposes, the cells were stained with Diff-Quik Solution I (American Scientific Products, Mcgrew Park, IL) for 10 min at 23°C. Solution I was removed, then Diff-Quik Solution II was added, and the cells were stained for another 10 min at 23°C. Wells were washed with PBS, and then representative cells were photographed with a Nikon DIAPHOT inverted phase microscope using Panatomic X film ASA32 (Eastman Kodak, Rochester, NY). Adherent cells to be quantitated for spreading were stained overnight at 23°C with 500 µl of a solution containing 0.12% (w/v) Coomasie brilliant blue R (Sigma Chemical Co.), 3% (v/v) acetic acid, and 50% (v/v) ethanol. Wells were washed three times with 500 µl of PBS, and cell spreading was then quantitated by measuring the average surface area occupied by a cell using an Opti-Max image analyzer. Thirty cells were measured per well; each experiment was done in quadruplicate and repeated three times.

Inhibition of cell adhesion with synthetic peptides or specific antibodies was performed similarly to previously described assays (11). Briefly, radiolabeled cells at 5 × 10^4/ml were incubated for 30 min with various concentrations of synthetic peptides or BSA in DMEM/Hepes containing 2 mg/ml BSA. The cell suspension (100 µl) was then added to wells precoated with 1.5 µg of laminin, 1.0 µg of type IV collagen, or 1.0 µg of fibronectin. The cells were incubated for 20 min at 37°C, the wells were washed, and adherent cells were quantitated as previously described (11). Cell viability after a 1-h incubation in the presence of the "inhibitors" was assessed by trypan blue dye exclusion. In all cases, the cells were >95% viable, and no toxicity of the peptides was observed at the indicated concentrations tested. In parallel studies, cells were incubated for 30 min with the peptides and then spun at 1000 rpm for 10 min to remove the nonadsorbed peptides. The cells were resuspended in DMEM/Hepes containing 2 mg/ml BSA and added to the protein-coated wells as described above. Similar levels of inhibition were observed irrespective of the presence of the peptides in the cell suspension added to the wells.

Alternatively, 100 µl of DMEM/Hepes containing 2 mg/ml BSA and various concentrations of the purified IgGs were added to wells which were precoated with 1 µg of peptide F-9, laminin, or fibronectin. The antibodies were incubated in the protein-coated wells for 1 h at 37°C. The cells were added and incubated for 30 min at 37°C, at which time the wells were washed and adherent cells quantitated as previously described (11). All experiments were repeated at least three times in quadruplicate.

Direct Cell Migration. Migration assays were performed in Boyden microchemotaxis chambers as recently described (46). For the chemotaxis experiments, various concentrations of laminin, fibronectin, synthetic peptides, or BSA were added to the lower compartments of the chambers and 50-µl aliquots of subconfluent fibrosarcoma cells at a concentration of 5-8 × 10^5 cells/ml in DMEM/Hepes were added to the upper compartments. Initially, cells were incubated at 37°C in a humidified incubator with 5% CO2 for various lengths of time ranging from 1 to 24 h; then the direct migration of the cells was quantitated. Maximal cell migration was observed after 6 h, so this time point was used in all subsequent migration assays.

Direct cell migration by haptotaxis was determined by precoating the filters overnight at 37°C on the top, bottom, or both surfaces in 100-µg/ml solutions of proteins or peptides in PBS, as described (46). Cells were incubated in the chambers for 6 h and then filters were quantitated for the migration of cells.

Inhibition of Laminin-mediated Cell Migration. Inhibition assays were designed whereby the migration of cells toward matrix molecules was monitored in the presence of peptides. Inhibition of cell migration by peptides was quantitated by coating the bottom surface of the filters in a 100-µg/ml solution of laminin or fibronectin. Cells were then incubated, in the continued presence of the peptides, for 6 h in the upper compartments of chambers which contained DMEM/Hepes in the bottom chamber.

A second series of inhibition assays was designed to study the inhibition of laminin-mediated cell migration by various antibodies. In this assay, the bottom surfaces of the filters were coated in 100-µg/ml solutions of laminin or fibronectin, as described above. Filters were then floated for 1 h at 37°C in 100-µg/ml solutions of sera from normal rabbits or rabbits immunized with laminin, peptide F-9, peptide CDPGYIGSR, or fibronectin. The filters were rinsed with PBS and placed in chambers containing DMEM/Hepes in the lower compartment, and cells in DMEM/Hepes were added to the upper compartment of triplicate chambers. Cell migration was quantitated after a 6-h incubation.

RESULTS

Cell Adhesion to Peptide F-9. Radiolabeled fibrosarcoma cells adhered in a proportionate manner to polystyrene plates coated with increasing concentrations of peptide F-9 (Fig. 1). Coating concentrations as low as 0.5 µg/well of peptide F-9 promoted cell adhesion above background levels (obtained on surfaces coated with BSA). Maximal adhesion of ~50% of the added cells occurred at coating concentrations of 5 µg/well of peptide F-9, after which point no further increase was observed. Two separate preparations of the synthetic peptide CDPGYIGSR were tested and neither promoted the adhesion of the fibrosarcoma cells, even at extremely high coating concentrations, such as 10 µg/well (Fig. 1). Two other synthetic peptides (F-11 and F-12) from the B1 chain of laminin (39) also failed to promote...
fibrosarcoma cells adhered in a concentration-dependent manner to wells coated with peptide F-9 (Fig. 3); however, only background levels of the cells (i.e., <10%) adhered to peptide CDPGYIGSR (Fig. 3) or peptides F-11 or F-12 (not shown). This finding suggests that the inability of the fibrosarcoma cells to recognize these other peptides is most likely not due to problems in the adsorption of the peptide to the surface used for the cell adhesion assay.

Morphologically, most of the fibrosarcoma cells which were incubated for 2 h on polystyrene plates coated with laminin spread out and had a flattened appearance (Fig. 4, a and b). The spreading of these cells was quantitated by measuring the average surface area occupied by a cell. Cells incubated on surfaces coated with laminin spread out to cover an average surface area of 443 \( \mu \text{m}^2 \) (Table 1). Fibrosarcoma cells incubated on surfaces coated with peptide F-9 also spread out and had a flattened appearance (Fig. 4, c and d). Quantitatively, the cells spread on peptide F-9 to occupy an average area of 286 \( \mu \text{m}^2 \) (Table 1); this area represents 60% of the spreading caused by intact laminin. The few fibrosarcoma cells which remained on the surfaces coated with peptide CDPGYIGSR (Fig. 4e), BSA (Fig. 4f), or peptides F-11 and F-12 (not shown) were spherical and did not flatten or spread out at all after 2 h; these cells only occupied 74 \( \mu \text{m}^2 \) (Table 1).

In order to ensure that the cell spreading promoted by peptide F-9 was not restricted to murine fibrosarcoma cells, other cell lines were also examined. Human cells of different histological origin were selected for this experiment: a human renal carcinoma cell line of low (SN12 C) and high (SN12 PM-6) metastatic capacity and normal human mesangial cells. In all cases, the cells adhered and spread on laminin and peptide F-9 (Table 1). Interestingly, irrespective of the cell type, the extent of cell spreading on peptide F-9 was ~60% of that observed on the intact laminin molecule.

Inhibition of Laminin-mediated Cell Adhesion. In order to determine whether the sequence of amino acids making up peptide F-9 were functionally active in the intact laminin molecule, radiolabeled cells were preincubated in various concentrations of synthetic peptides or BSA and then added to wells coated with laminin, type IV collagen, or fibronectin. Exogenous peptide F-9 inhibited laminin-mediated cell adhesion in a similar to peptide CDPGYIGSR and BSA (data not shown). Points, means of four separate determinations; the SEM was <5% in each case. Three separate experiments gave similar results.

Radiolabeled fibrosarcoma cells adhered to peptide F-9 in a time-dependent manner (Fig. 2). When polystyrene plates were coated with 1 \( \mu \text{g} \) of peptide F-9, significant cell adhesion was observed over background levels as rapidly as 15 min after the cells were added. By 30 min, one-half of the maximal cell adhesion occurred, and by 2 h, maximal cell adhesion levels were observed. The adhesion of fibrosarcoma cells to laminin-coated surfaces followed a very similar time course (Fig. 2). Peptide CDPGYIGSR (Fig. 2), peptides F-11 and F-12 (not shown), and BSA (Fig. 2) did not promote cell adhesion, even after a 3 h incubation period.

To ensure that the peptides had adsorbed to the plastic wells, we radiolabeled the peptides with \(^{1}H\)formaldehyde and quantitated the amount of peptide bound to the wells. When equimolar coating concentrations of the peptides were used, the peptides adsorbed to the wells coated with the peptides (Fig. 3); however, only background levels of the cells (i.e., <10%) adhered to peptide CDPGYIGSR (Fig. 3) or peptides F-11 or F-12 (not shown). This finding suggests that the inability of the fibrosarcoma cells to recognize these other peptides is most likely not due to problems in the adsorption of the peptide to the surface used for the cell adhesion assay.

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![Graph](image-url)

**Protein or Peptide Concentration (\( \mu \text{g/well} \))**

Fig. 1. Adhesion of fibrosarcoma cells to increasing coating concentrations of proteins or peptides. Cells were radiolabeled and incubated for 2 h at 37°C in microtiter wells coated with various concentrations of peptide F-9 (D), laminin (.), peptide CDPGYIGSR (\( \Delta \)), or BSA (\( \Delta \) as described in "Materials and Methods." Control peptides F-11 and F-12 did not promote cell adhesion, similar to peptide CDPGYIGSR and BSA (data not shown). Points, means of four separate determinations; the SEM was <5% in each case. Three separate experiments gave similar results.

![Graph](image-url)

**Time (hr)**

Fig. 2. Time course of the adherence of fibrosarcoma cells to surfaces coated with proteins or peptides. Cells were radiolabeled and incubated for increasing periods of time at 37°C in microtiter wells coated with 1 \( \mu \text{g} \) of peptide F-9 (D), laminin (.), peptide CDPGYIGSR (\( \Delta \)), or BSA (\( \Delta \) as described in "Materials and Methods." Control peptides F-11 and F-12 did not promote cell adhesion, similar to peptide CDPGYIGSR and BSA (data not shown). Points, means of four separate determinations; the SEM was <5% in each case. Three separate experiments gave similar results.

![Graph](image-url)

**Peptide Adsorbed (pmoles)**

Fig. 3. Adhesion of fibrosarcoma cells to increasing concentrations of adsorbed peptides. Cells were radiolabeled and incubated for 2 h at 37°C in microtiter wells coated with equivalent concentrations of peptides (quantitated in pmoles): peptide F-9 (D) or peptide CDPGYIGSR (\( \Delta \) as described in "Materials and Methods," Points, means of four separate determinations; the SEM was <5% in each case. Two separate experiments gave similar results.
LAMININ PEPTIDE CAUSES FIBROSARCOMA CELL ADHESION

Fig. 4. Spreading of fibrosarcoma cells on surfaces coated with proteins or peptides. Cells were incubated for 2 h at 37°C in microtiter wells coated with 1 µg of laminin (a and b), peptide F-9 (c and d), peptide CDPGYIGSR (e), or BSA (f). The wells were then washed with DMEM/Hepes containing 2 mg/ml BSA to remove nonadherent cells and then photographed as described in “Materials and Methods.” Bar, 50 µm. Three separate experiments gave similar results.

Table 1 Cell adhesion and spreading

| Surfaces were coated with proteins or peptides at 5 µg/well and cells were incubated for 2 h at 37°C. |
|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|
| Cell lines                                      | Adhesion*                                        | Spreading*                                       |
|                                                  | Laminin  | F-9       | BSA     | Laminin  | F-9       | BSA     |
| UV-2237-MM murine fibrosarcoma                   | 43 ± 7   | 52 ± 5    | 1 ± 0.1 | 443 ± 32  | 286 ± 11  | 74 ± 6  |
| SN12 C human renal carcinoma (low metastatic)    | 28 ± 4   | 54 ± 11   | 1 ± 0.1 | 520 ± 41  | 300 ± 16  | 75 ± 8  |
| SN12 PM-6 human renal carcinoma (high metastatic)| 36 ± 7   | 56 ± 9    | 1 ± 0.2 | 550 ± 29  | 310 ± 12  | 75 ± 5  |
| Normal human mesangial cells                     | 91 ± 2   | 73 ± 5    | 1 ± 0.3 | 1042 ± 51 | 637 ± 31  | 92 ± 11 |

*Adhesion is quantitated as a percentage of the total cells added. Each value represents the mean of four separate determinations ± SEM.

*Spreading is quantitated as the average surface area occupied by a cell in µm². Each value represents the mean of 30 separate cell measurements ± SEM and was done in duplicate. The values reported for cell spreading on BSA were similar to those observed on peptides CDPGYIGSR or peptides F-11 and F-12.

concentration-dependent manner, and at 40 pmol, peptide F-9 inhibited >70% of the cell adhesion to laminin (Fig. 5) but had no effect on cell adhesion to type IV collagen or fibronectin (not shown). No inhibition of cell adhesion toward laminin or fibronectin was observed when cells were preincubated with peptide CDPGYIGSR, peptides F-11 or F-12, or BSA.

Polyclonal antibodies were raised in rabbits against peptide F-9 or peptide F-9/KLH and the specificity of the purified IgG was verified by RIA. Anti-peptide F-9 IgG reacted specifically with peptide F-9 and laminin (Fig. 6). At concentrations as low as 0.01 or 1 µg/ml the anti-peptide F-9 IgG still recognized peptide F-9 or laminin, respectively. The titer of anti-peptide F-9/KLH IgG was similar to that of the anti-peptide F-9 IgG, so both IgG sources were used in these studies. No reactivity was observed on surfaces coated with peptides CDPGYIGSR (Fig. 6), fibronectin (Fig. 6), or peptides F-11, F-12, or BSA (not shown).

In a second competition assay, polyclonal antibodies produced against peptide F-9 were tested for the ability to inhibit the adhesion of fibrosarcoma cells to peptide F-9, laminin, or fibronectin. In these studies, various concentrations of purified anti-peptide F-9 IgG were incubated in wells precoated with peptide F-9, laminin, or fibronectin, and then radiolabeled fibrosarcoma cells were added. Final IgG concentrations as low
cells were preincubated for 30 min in various concentrations of peptide F-9 (D), peptide CDPGYIGSR (Δ), peptide F-11 (○), or peptide F-12 (●), and then added to wells coated with 1.5 µg of laminin. After a 20-min incubation, the nonadherent cells were aspirated, and the wells were washed three times. Adherent cells were quantitated in a scintillation counter. Cell inhibition is expressed as a percentage relative to the number of cells binding to laminin-coated surfaces in the absence of inhibitors. Points, means of four separate determinations; bars, ± SEM. Three separate experiments gave similar results.

Fig. 5. Inhibition of laminin-mediated cell adhesion by peptides. Radiolabeled cells were preincubated for 30 min in various concentrations of peptide F-9 (○), peptide CDPGYIGSR (Δ), peptide F-11 (○), or peptide F-12 (●), and then added to wells coated with 1.5 µg of laminin. After a 20-min incubation, the nonadherent cells were aspirated, and the wells were washed three times. Adherent cells were quantitated in a scintillation counter. Cell inhibition is expressed as a percentage relative to the number of cells binding to laminin-coated surfaces in the absence of inhibitors. Points, means of four separate determinations; bars, ± SEM. Three separate experiments gave similar results.

Fig. 6. Specificity of purified polyclonal IgG against peptide F-9. Microtiter wells were coated in triplicate with 1 µg of peptide F-9 (○), laminin (●), peptide CDPGYIGSR (Δ), or fibronectin (□). Increasing concentrations of purified IgG against peptide F-9 were added to the wells, followed by 125I-donkey anti-rabbit IgG as described in "Materials and Methods." Background binding to each antigen using normal rabbit IgG has been subtracted from each value, with the SEM ≤ 5%. No reactivity was observed when peptides F-11 or F-12 were used as the antigen in the RIA.

Fig. 7. Inhibition of fibrosarcoma cell adhesion to peptide F-9 and intact laminin by polyclonal antibodies against peptide F-9. Microtiter wells were coated with peptide F-9 (○), laminin (●), or fibronectin (□). Purified IgG (either from normal rabbits (○, ●, ○) or rabbits immunized with peptide F-9 (●, ●, ●)) were incubated for 1 h at 37°C in the wells and then radiolabeled cells were added as described in "Materials and Methods." The percentage of inhibition of cell adhesion was quantitated as [1 - (the number of cells bound in the presence of the inhibitor + the number of cells bound in the absence of the inhibitor)] × 100. Points, the means of four separate determinations; bars, ± SEM. Three separate experiments gave similar results.

Fig. 8. Direct migration of fibrosarcoma cells to laminin. Various concentrations of laminin (●) or BSA (○) were added to the lower compartments of the microchemotaxis chambers. Cells were then added to the upper compartments as described in "Materials and Methods." After a 6-h incubation at 37°C, filters were washed, fixed, and stained and the cells which migrated to the bottom side of the filters were quantitated. Cells in four randomly selected high power fields were quantitated for each compartment. Points, mean numbers of migrated cells/mm² for three separate compartments; bars, ± SEM. Since the surface area of the filter is 7 mm², the total number of cells migrated is seven times the number presented on the y axis.

as 10 µg/ml inhibited fibrosarcoma cell adhesion to peptide F-9 and laminin about 30%, and as the IgG concentration was increased, the inhibition of cell adhesion to laminin or peptide F-9 increased up to ~70 and ~90%, respectively (Fig. 7). Anti-peptide F-9 IgG did not inhibit cell adhesion to fibronectin. Inhibition of cell adhesion to peptide F-9, laminin, or fibronectin caused by normal rabbit IgG was always < 5%.

Direct Cell Migration to Laminin. Fibrosarcoma cell migration was monitored in the presence of increasing levels of soluble laminin below the filter. Cells migrated in a concentration-dependent manner when increasing concentrations of laminin were added to the lower compartments of the chambers (Fig. 8). At concentrations as low as 10 µg/ml, laminin was able to stimulate fibrosarcoma cell migration 7-fold greater than the background levels observed with BSA (350 and 50 cells/mm², respectively). When the concentration of laminin was increased to 100 µg/ml, ~820 cells/mm² migrated and adhered onto the underside of the filter. Since the area of each filter is 7 mm², the total number of cells migrated is seven times the number presented on the y axis.

In previous studies (46, 54–56), the migration of cells in Boyden microchemotaxis chambers was found to be due to haptotaxis, i.e., the generation of a substratum-bound density gradient of protein adsorbed to the bottom surface of the filter. Fibrosarcoma cells migrated on filters precoated on the bottom surface with laminin and no additional soluble laminin was required to be present in the lower compartment below the filter for cell migration to occur. The number of cells which migrated toward the filters which had been precoated in 100-µg/ml solutions of laminin was similar to the number of cells which migrated in response to 100-µg/ml solutions of laminin present in the lower compartment (~800 cells/mm²). This
Effect of peptide F-9 on laminin-mediated cell migration, poly-

to the upper compartments of the chambers. Cell migration was quantitated as described above in Fig. 8. Peptides II1 and II.' had no effect on laminin-mediated cell migration (not shown).

The inhibitory effect of peptide F-9 was specific to laminin since it mediated cell migration to decrease almost 80% (Fig. 9). The inhibitory effect of peptide F-9 was specific to laminin since it had no effect on cell migration toward fibronectin (data not shown). Preincubation of cells with peptides CDPGYIGSR (Fig. 9), F-l 1, F-12 (not shown), or BSA (Fig. 9) had no effect on cell migration toward either laminin or fibronectin (not shown), even at concentrations as high as 100 µg/ml.

Inhibition of Laminin-mediated Cell Migration by Peptides. Laminin-mediated cell migration was monitored by preincubating cells with exogenous synthetic peptides before adding them to the upper compartments of the chambers. Preincubation of cells with as little as 10 µg/ml of peptide F-9 caused ~40% inhibition of laminin-mediated cell migration, and increasing the concentration of peptide F-9 to 100 µg/ml caused laminin-mediated cell migration to decrease almost 80% (Fig. 9). The inhibitory effect of peptide F-9 was specific to laminin since it had no effect on cell migration toward fibronectin (data not shown). Preincubation of cells with peptides CDPGYIGSR (Fig. 9), F-11, F-12 (not shown), or BSA (Fig. 9) had no effect on cell migration toward either laminin or fibronectin (not shown), even at concentrations as high as 100 µg/ml.

Inhibition of Laminin-mediated Cell Migration by Antibodies against Peptide F-9. In order to further examine the inhibitory effect of peptide F-9 on laminin-mediated cell migration, polyclonal antibodies raised against peptide F-9 were tested in competition assay.

Filters were coated on the bottom surface in 100-µg/ml solutions of either laminin or fibronectin as described for the haptotaxis experiments above; then the coated filters were incubated in 100-µg/ml solutions of sera from normal rabbits or rabbits immunized with laminin, fibronectin, peptide F-9, or peptide CDPGYIGSR. Cell migration toward laminin was inhibited by ~90% when filters were incubated in sera from rabbits immunized with either laminin or peptide F-9 (Fig. 10). In contrast, cell migration toward laminin was not affected when the filters were incubated in sera from normal rabbits or rabbits immunized with peptide CDPGYIGSR or fibronectin (Fig. 10). As a control, cell migration toward fibronectin was shown to decrease only in the presence of IgG against fibronectin, with no inhibition of cell migration toward fibronectin in the presence of IgG against laminin, peptide F-9, normal rabbit, or no IgG (not shown).

Migration of Cells to Synthetic Peptides. Since exogenous peptide F-9 and antibodies against peptide F-9 inhibited laminin-mediated cell migration, we next tested peptide F-9 for the ability to stimulate the directional migration of fibrosarcoma cells in microchemotaxis chambers. Peptide F-9, when present in solution or coated to the bottom of filters, did not directly promote cell migration over a concentration range of 1-100 µg/ml (not shown). Although previous studies had shown that the synthetic peptide CDPGYIGSR stimulated the migration of B16F10 melanoma cells (44), it did not stimulate fibrosarcoma cell migration even at concentrations as high as 100 µg/ml. No increase in cell migration was observed when increasing concentrations of peptides F-11, F-12, or BSA were present in the lower compartments (not shown).

Effect of Partial Sequences of Peptide F-9 on Cell Adhesion. In order to further define the structural features of peptide F-9 which are important for promoting cell adhesion activity, portions of peptide F-9 were synthesized and HPLC purified (Table 2). Initially, five peptides, encompassing various regions of peptide F-9 were synthesized, each of which retained at least two positively charged amino acids (arginine or lysine). These peptides were adsorbed on polystyrene plates as described above, and fibrosarcoma cell adhesion was quantitated after a 2-h incubation. The amino terminal two-thirds of peptide F-9 [1-13] was as active as intact peptide F-9 at promoting cell adhesion, while the amino terminal one-third of peptide F-9 [1-7] had about one-half of the activity (Fig. 11). In addition, the middle one-third of peptide F-9 [7-13], the carboxy terminal two-thirds of peptide F-9 [7-20], and the carboxy terminal one-third of peptide F-9 [13-20] were all incapable of promoting cell adhesion (Fig. 11). These results suggest that the cell adhesion-promoting activity of peptide F-9 resides in the amino terminal one-third region of the peptide.

An inhibition assay was performed to assure that the peptides which directly promoted fibrosarcoma cell adhesion could also inhibit laminin-mediated or peptide F-9 [1-20]-mediated cell adhesion. Based on the direct cell adhesion assays (Fig. 11), which suggested that the active region of peptide F-9 was comprised of the amino terminal residues, RYVVLPVR, 11 additional peptides were synthesized encompassing smaller portions of peptide F-9 (Table 2). Since these peptides may not
LAMININ PEPTIDE CAUSES FIBROSARCOMA CELL ADHESION

Table 2  Inhibition of fibrosarcoma cell adhesion to laminin or peptide F-9 by partial sequences of peptide F-9

<table>
<thead>
<tr>
<th>Peptide name&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Amino acid sequences</th>
<th>Inhibition of cell adhesion&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-9[1-7]</td>
<td>R Y V V L P R</td>
<td>++</td>
</tr>
<tr>
<td>F-9[7-20]</td>
<td>R V C F E K G M N Y T V R</td>
<td>--</td>
</tr>
<tr>
<td>F-9[7-13]</td>
<td>R V C F E K</td>
<td>--</td>
</tr>
<tr>
<td>F-9[1-12]</td>
<td>R Y V V L P R P V C F E</td>
<td>+++</td>
</tr>
<tr>
<td>F-9[1-8]</td>
<td>R Y V V L P R</td>
<td>+</td>
</tr>
<tr>
<td>F-9[1-5]</td>
<td>R Y V V L</td>
<td>+</td>
</tr>
<tr>
<td>F-9[1-4]</td>
<td>R Y V V</td>
<td>+</td>
</tr>
<tr>
<td>F-9[1-3]</td>
<td>R Y V</td>
<td>+</td>
</tr>
<tr>
<td>F-9[2-7]</td>
<td>Y V V L P R</td>
<td>+</td>
</tr>
<tr>
<td>F-9[3-13]</td>
<td>V V L P R P V C F E</td>
<td>+</td>
</tr>
<tr>
<td>F-9[3-11]</td>
<td>V V L P R P V C</td>
<td>+</td>
</tr>
<tr>
<td>F-9[3-7]</td>
<td>V V L P R</td>
<td>+</td>
</tr>
<tr>
<td>F-9[4-7]</td>
<td>V V L P</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup>The numbers in brackets refer to the consecutive amino acid numbers as they appeared in the original 20 amino acids from peptide F-9.

<sup>b</sup>Cell adhesion to laminin or peptide F-9 was studied in the presence of exogenous soluble peptides at 100 μg/ml. Inhibition of cell adhesion was quantitated relative to the inhibition observed in the presence of exogenous peptide F-9 [1-20] and scored as follows: ++++, inhibition comparable to exogenous peptide F-9 [1-20]; ++, inhibition 50-75% of that observed with exogenous peptide F-9 [1-20]; +, inhibition 25-50% of that observed with exogenous peptide F-9 [1-20]; -, no inhibition.

DISCUSSION

In this study, synthetic peptide F-9 from the B1 chain of laminin directly promoted the adhesion and spreading of a metastatic murine fibrosarcoma cell line in a time- and concentration-dependent manner. Peptide F-9, as well as polyclonal antibodies against peptide F-9, inhibited cell adhesion to laminin but not fibronectin or type IV collagen, providing further evidence that peptide F-9 is a major cell adhesion-promoting sequence in intact laminin. Laminin promoted the migration of these cells in a concentration-dependent fashion. Additionally, peptide F-9 and polyclonal antibodies against peptide F-9 inhibited laminin-mediated cell migration. Peptide F-9 functions as a domain of laminin to which cells adhere, but precisely how it plays a role in cell migration is not clear. This is shown by the ability of peptide F-9 or anti-peptide F-9 IgG to inhibit laminin-mediated cell migration, yet peptide F-9 by itself cannot directly promote cell migration. If cell migration is viewed as a multistep process whereby cells first must adhere to the "attractant," spread, and then be released from the attractant and move onward, our findings with peptide F-9 suggest that it mediates the initial stage of migration, i.e., adhesion to the surface attractant. Perhaps in the inhibition assays peptide F-9 binds to a receptor for laminin on the cell surface and blocks the adhesion of the cell to laminin so that the cell can no longer adhere, spread, and migrate on laminin. Importantly, these results with peptide F-9 also indicate that the process of cell adhesion is necessary but not sufficient for cell movement to occur as shown for other proteins in previous studies (46, 47, 57).

In order to further elucidate the region of peptide F-9 which promotes cell adhesion, peptide F-9 was synthesized in smaller portions of peptide F-9 inhibited cell adhesion to fibronectin or type IV collagen (not shown).
portions. The amino terminal two-thirds of peptide F-9 directly promoted cell adhesion in levels equivalent to intact peptide F-9, and direct cell adhesion-promoting activity was further localized to the first seven amino acids, RYVVLPR. The residues RYVV from the amino terminus were determined to be the minimal sequence of peptide F-9 capable of inhibiting laminin- or peptide F-9-mediated fibrosarcoma cell adhesion. The fact that the amino acid sequence of the B1 chain of laminin derived from the murine EHS tumor comprising peptide F-9 is identical except for one amino acid to the sequence from human laminin (the 15th residue is a threonine in the human and a methionine in the murine) further suggests its importance in the adhesion of cells of human origin. Furthermore, this one altered residue is not present in the functionally active region of peptide F-9. Interestingly, the peptide F-9 sequence from murine laminin is 50% homologous to the equivalent sequence in Drosophila (58); this is significant when one considers that this domain of laminin only has ~25% amino acid identity between Drosophila and mouse. In addition, three of the four amino terminal amino acids which contain biological activity, RYVV, are identical to those in Drosophila, RQVV.

Since the first seven amino acids of peptide F-9 directly promoted fibrosarcoma cell adhesion, a homology search for the sequence RYVVLPR was performed by computer against all known protein and peptide sequences. No proteins or peptides were found to contain these same seven residues or even six of the seven. However, five proteins contained five of the seven amino acids. These five proteins were the hexon-associated protein (VIII) from mastadenovirus h2 (59, 60), hexonines B and PII from the yeast Saccharomyces cerevisiae (61, 62), phosphoribosylamine-glycine ligase from Bacillus subtilis (63), and human angiotensinogen precursor (64, 65). The five amino acids which are shared by peptide F-9 and these proteins are VVLP; however, the active site of peptide F-9, RYVV, was not in any other molecules and appears to be unique to laminin peptide F-9.

Interestingly, peptide CDPGYIGSR from the B1 chain of laminin did not promote the adhesion or migration (chemotaxis or haptotaxis) of the metastatic murine fibrosarcoma cells used in this study. Furthermore, neither peptide CDPGYIGSR nor antibodies against peptide CDPGYIGSR were capable of inhibiting laminin-mediated cell adhesion or migration. Previous studies have reported that this peptide promotes the adhesion of various cell lines, including HT-1080 human fibrosarcoma cells (41, 42) and the migration of B16F10 melanoma cells (44). It is possible that the fibrosarcoma cell line used in our studies utilizes different receptors to adhere to laminin than those cells studied by others. We have noticed this same phenomenon when we tested other cell lines. For example, K-1735-M4 murine melanoma and PC12 rat pheochromocytoma cells did not adhere to peptide CDPGYIGSR but did adhere to peptide F-9.4 Therefore, cell-specific domains on laminin recognized by multiple cell surface receptors may be responsible for the differences observed in the adhesion and migration of the various cell types.

Several domains of laminin have been described which promote the adhesion of various cell types. For example, while certain tumor cells adhere to the intersection of the cross (6, 7), other tumor cells adhere to a domain(s) below the intersection of the cross (11). Additionally, rat hepatocytes adhere and spread on the short arm of laminin opposite the long arm (7), and neurite outgrowth and myoblast adhesion occurs on the distal part of the long arm of laminin (8–10). Based on the large size of laminin (850 kDa), it is clear that multiple domains could be present which would promote cell adhesion. This is supported by our finding that peptide F-9 directly promotes cell adhesion, but, when present in solution, peptide F-9 cannot completely inhibit laminin-mediated cell adhesion, perhaps due to the presence of additional cell adhesion-promoting domains or sequences in laminin.

Peptide F-9 promotes the adhesion of cells of quite different histological origin, species, and metastatic potential: from highly metastatic murine melanoma and fibrosarcoma cells to C6 rat glioma cells, PC12 rat pheochromocytoma cells, and bovine aortic endothelial cells (39). Peptide F-9 also promotes the adhesion of human melanoma cells of high (A375SM) and low metastatic capacity (A375P), as well as HT-1080 human fibrosarcoma cells.4 In this study, we have shown that peptide F-9 can also promote the adhesion and spreading of cells, in particular UV-2237-MM murine fibrosomas, human renal carcinomas, and normal human mesangial cells. It will be important to isolate and characterize a cell surface receptor for this peptide, especially since other laminin receptors have been described. It is unlikely that the fibrosarcoma cell surface receptor for peptide F-9 is the previously described 67-kDa laminin receptor since peptide CDPGYIGSR has already been reported to bind to that receptor (42) and these cells do not recognize peptide CDPGYIGSR. Integrin receptors which bind to laminin have been isolated from certain cell types (26, 28–31). Recently, an α3β1 integrin isolated from osteosarcoma cells has been reported to bind near the carboxy terminus of the B1 chain of laminin (27). Peptide F-9 may bind to yet another receptor, perhaps a proteoglycan, since peptide F-9 has been shown to specifically bind heparin (39). Other cell surface receptors for laminin ranging in molecular size from 56 to 180 kDa (22–25) have been isolated from various cell lines. Future studies will be required in order to determine whether peptide F-9 recognizes any of these known receptors.

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Definition of a Sequence, RYVVLPR, within Laminin Peptide F-9 that Mediates Metastatic Fibrosarcoma Cell Adhesion and Spreading

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